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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

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TITLE OF THE INVENTION (500 characters max)

Demonstration of Streptococcus Mutans with Specific Cell Wall Polysaccharide

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ENCLOSED APPLICATION PARTS (check all that apply)

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METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (check one)

☐ Applicant claims small entity status. See 37 C.F.R. § 1.27. ☒ A check or money order is enclosed to cover the Provisional filing fees. ☒ The Director is hereby authorized to charge any deficiency in filing fees or credit any overpayment to Deposit Account No. 02-4800. This paper is submitted in duplicate.	**PROVISIONAL FILING FEE AMOUNT(S)**	☐ **$80.00 (2005)** ☒ **$160.00 (1005)**

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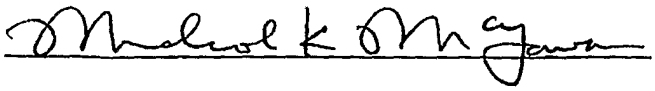
Respectfully submitted,

SIGNATURE  DATE December 30, 2003

TYPED or PRINTED NAME Malcolm K. McGowan, Ph.D. Registration No. 39,300 *(if appropriate)*

[0001] **DEMONSTRATION OF A NEW SEROTYPE OF STREOTOCOCCUS MUTANS WITH SPECIFIC CELL WALL POLYSACCHARIDE IN THE HUMAN ORAL CAVITY AND A METHOD FOR DETECTION THEREOF**

[0002] **INTRODUCTION**

[0003] Based on their differences in composition and the linkage of cell wall polysaccharides, mutans streptococci are classified into 8 serotypes as follows; *Streptococcus mutans* (serotype *c*, *e*, *f*), *Streptococcus sobrinus* (*d*, *g*), *Streptococcus cricetus* (*a*), *Streptococcus rattus* (*b*), *Streptococcus ferus* (*c*), *Streptococcus macacae* (*c*), and *Streptococcus downei* (*h*) (3). Among these, *S. mutans* is known to be a major causative bacterium of dental caries in humans, and is occasionally isolated from the blood of patients with infective endocarditis (IE) (19, 20). In our previous study, 4 streptococcal strains isolated from patients with IE or bacteremia following a tooth extraction procedure were specified as *S. mutans* based on their biological properties and 16S ribosomal RNA (rRNA) alignment (4). However, 2 of these strains (TW295 and TW871) were shown to be serologically untypable, while the others belonged to serotype *e* or *f*.

[0004] The serotype specific polysaccharides of *S. mutans* are composed of rhamnose-glucose polymers, with a backbone of rhamnose and side chains of α- or β-linked glucosidic residues (9). The serologically untypable properties in strains of TW295 and TW871 have been shown to be derived from the lack of a glucose side chain in the serotype specific polysaccharide (4). Further, the polysaccharides of these blood isolates share similarities with those of the mutant strain with inactivation of *gluA* gene, which encodes the enzyme that catalyzes the production of the immediate precursor of the glucose side chain donor, in that they all had low glucose contents (21).

[0005] The serologically untypable isolate strains were shown to have hydrophobicity and sucrose-dependent adhesion at levels as high as the oral isolates (4), and were less susceptible to phagocytosis in a preliminary study. These findings imply

that *S. mutans* organisms without a glucose side chain of serotype *c*, *e*, or *f* specific polysaccharide may be present in the oral cavity of humans, because of their high cellular hydrophobicity and sucrose-dependent adherence levels. Further, they may be able to survive in blood longer owing to their low susceptibility to phagocytosis during invasion of blood. In the present study, we surveyed the occurrence of *S. mutans* organisms from human oral cavities that had similar serological properties as untypable blood isolates.

[0006] EXAMPLE 1

[0007] MATERIALS & METHODS

[0008] ***S. mutans* strains.** Blood isolates TW295 (untypable), TW871 (untypable), TW964 (*f*), and TW1378 (*e*) (4), and orally isolated strains MT8148 (*c*), MT4245 (*e*), and MT4251 (*f*) were selected from the stock culture collection in our laboratory (14). Features of these strains are presented in Table 1.

[0009] TABLE 1. Bacterial strains used in the present study

Strain	Serotype	Features	Reference
TW295	*k*	Blood isolate from a 59-year-old male with bacteremia following a tooth extraction procedure	4
TW871	*k*	Blood isolate from a 45-year-old female with infective endocarditis complicated with subarachnoid hemorrhage	4
TW964	*f*	Blood isolate from a 72-year-old male with infective endocarditis	4
TW1378	*e*	Blood isolate from a 59-year-old male with infective endocarditis	4
MT8148	*c*	Oral isolate from a healthy child	13
MT4245	*e*	Oral isolate from a healthy child	13
MT4251	*f*	Oral isolate from a healthy child	13

Strain	Serotype	Features	Reference
NN2001	*c*	Oral isolate from a 6-year-old healthy boy	This study
NN2002	*e*	Oral isolate from a 9-year-old healthy boy	This study
NN2003	*f*	Oral isolate from a 7-year-old healthy girl	This study
MT8148GD	*k*	Glucose side chain defective mutant strain of MT8148	This study
FT1 (NN2011)	*k*	Oral isolate from a 3-year-old healthy girl	This study
SU1 (NN2029)	*k*	Oral isolate from a 10-year-old healthy girl	This study
YK1	*k*	Oral isolate from a 6-year-old girl with Down's syndrome	This study

[0010] **Generation of antisera against untypable *S. mutans* cells.** Antisera to serotypes *c*, *e*, and *f* of *S. mutans* were taken from our laboratory stock (12), while those to untypable strains TW295 and TW871 were generated as follows. Whole cells of each strain were suspended in phosphate-buffered saline (PBS), and injected intravenously into rabbits repeatedly for 5 consecutive days (dry weight; 5 mg per day). One week after the final injection, immunization was repeated another 2 weeks, 5 times each week. Blood was then taken from the auricular vein and the antibody titer was checked using an immunodiffusion method with the Rantz-Randall (RR) polysaccharide antigen (5) to TW295 or TW871.

[0011] **Construction of glucose side chain defective mutant strain.** A glucose side chain defective (GD) mutant strain of MT8148 was constructed by insertional inactivation of the *gluA* gene. The *gluA* gene and its flanking region were amplified by polymerase chain reaction (PCR) using AmpliTaq Gold polymerase (Applied Biosystems, Foster City, Calif.), with primers constructed on the basis of the *gluA* sequence from *S. mutans* strain Xc (21) (Genbank accession no. AB001562) and the *S. mutans* genomic database published by Oklahoma University (1) (Genbank accession no. AE014133). The amplified fragment was then cloned into a pST Blue-1 vector

(Promega, Madison, Wis.) to generate pRN101. The middle of the open reading frame of *gluA* in pRN101 was cleaved by *Stu* I, then ligated with the erythromycin resistant gene (*erm*) from recombinant plasmid pKN100 carrying an 830 bp fragment of *erm* from pVA838 (10) to yield pRN102. After linearization by digestion at the unique restriction enzyme site of *Not* I, the plasmid was introduced into *S. mutans* MT8148 by the method of Tobian and Macrina (18). The transformants were screened on Mitis-salivarius (MS) agar (Difco Laboratories, Detroit, Mich.) plates containing erythromycin (10 μg/ml). The appropriate insertional inactivation of the mutant MT8148GD was confirmed by PCR amplification of the *gluA* gene and immunodiffusion method.

[0012] **Clinical specimens.** One thousand three hundred twenty-six *S. mutans* strains isolated from 571 children between 1982 and 1990 (MT4000 or MT10000 series of isolates) were selected from our laboratory stock. In addition, strains from 100 subjects (3 to 17 years of age; average 8.9 years old) who visited the Pedodontics Clinic of Osaka University Dental Hospital, Suita, Osaka, Japan, from June to August in 2002 were randomly selected (the NN2000 series of isolates). These subjects included 88 healthy children along with 5 patients with cleft lip and palate, 3 with ventricular septal defect, 2 with amelogenesis imperfecta, 1 with spondyloschisis, and 1 autistic patient. Collection of clinical specimens was carried out in accordance with the Osaka University Health Guideline for Studies Involving Human Subjects. Plaque samples were collected in sterile PBS, diluted and streaked onto MS agar (Difco) plates containing bacitracin (0.2 units/ml; Sigma Chemical Co., St. Louis, Mo.) and 15% (w/v) sucrose. One colony from each subject was chosen on the basis of colonial morphology and defined as *S. mutans* as described previously (4). Serotyping was determined by the immunodiffusion method as described above, using rabbit antisera specific for serotypes *c*, *e*, and *f* (12). In addition, 50 colonies were isolated from each subject with a serologically untypable strain.

[0013] Two thousand five hundred clinical isolates of *S. mutans* were obtained from another group of 50 subjects (3 to 19 years of age; average 7.8 years old) who came to

the Pedodontics Clinic of Osaka University Dental Hospital in early 2003. These included 45 healthy children and 5 patients with general or oral health problems such as Down's syndrome, congenital heart disorder, cleft lip and palate, amelogenesis imperfecta, and oligodontia.

[0014] **Characterization of untypable *S. mutans*.** Sucrose-dependent adhesion and cellular hydrophobicity of the untypable clinical isolates were evaluated as described by Hamada *et al.* (6), and Rosenberg *et al.* (17), respectively. The expressions of cell-associated (CA-) or cell-free (CF-) glucosyltransferases (GTFs) and the surface protein antigen (PA) were analyzed using Western blot analysis with antibodies specific to GTF and PA (4). Genomic DNA was extracted from the test organisms, and 16S rRNA was sequenced and compared with that of reference strain NCTC10449 (2) (GenBank accession no. X58303, S70358).

[0015] **Phagocytosis assay.** Organisms were cultured in Brain Heart Infusion broth (Difco) for 18 h at 37°C. After washing the bacterial cells, cell concentrations were adjusted with PBS to 1.0×10^8 CFU/ml. Human peripheral blood (500 μl) was collected from a healthy volunteer and incubated with 500 μl (5.0×10^7 CFU) of the tested bacteria for 10 min at 37°C. Interactions between polymorphonuclear leukocytes (PMNs) and bacteria were observed following Giemsa staining (Wako Pure Chemical Industries, Osaka, Japan) and a light microscope (magnification, x100; Olympus Optical, Tokyo, Japan). The rate was expressed by the mean ratio of phagocytosed PMNs per 100 PMNs, with 500 PMNs examined. In addition, changes in the rate of phagocytosis in strains MT8148, MT8148GD, FT1, and TW295 were observed at 15, 30, 60, 90, and 120 min.

[0016] **Statistical analysis.** Intergroup differences of various factors were estimated by a statistical analysis of variance (ANOVA) for factorial models. Fisher's protected least-significant difference test was used to compare individual groups.

[0017] **RESULTS**

[0018] **Serotype distribution of the past and recent strains.** It was found that the antisera to TW295 and TW871 reacted specifically with RR extracts of TW295 and

TW871, respectively, however, not with those from serotype *c*, *e*, or *f* organisms. Further, the precipitation bands were fused to each other. Our laboratory records indicate that all of the 1326 strains from 571 children (MT series) isolated between 1982 and 1990 were serologically classified as *c*, *e*, or *f*, and with no untypable isolates found. On the other hand, 78, 17, and 3 of the recent 100 isolates (NN 2000 series) from 100 subjects were classified as serotype *c*, *e*, and *f*, respectively, whereas the remaining 2 strains, FT1 (NN2011) and SU1 (NN2029), were found to be not react with *c*, *e*, or *f* specific antiserum (Table 2), though both showed rough colonies on MS agar plates, positive bacitracin resistance, γ hemolysis on blood agar, a positive fermentation profile for mannitol, sorbitol, raffinose, and melibiose, and a negative dextran agglutination, which are characteristics of *S. mutans*. Moreover, they also expressed PA and three types of GTFs.

[0019] TABLE 2. Serotype distribution in 2 series of clinical oral isolates of *S. mutans*

Serotype	MT series taken between 1982 and 1990 (N=1326)	NN2000 series taken in 2002 (N=100)
c	1131	78
e	163	17
f	32	3
k	0	2

[0020] Strains FT1 and SU1 showed high levels of sucrose-dependent adhesion and cellular hydrophobicity (data not shown), and their 16S rRNA gene sequences were identical to that of strain NCTC10449 (Genbank accession no. X58303 and S70538). In addition, the RR extracts of strains FT1 produced a precipitation band with antisera to strain TW871 and TW295, which also fused to a precipitation band of the RR extract of TW871 with antiserum to TW871. Furthermore, fifty strains from each subject with strain FT1 or SU1 were not reactive with *c*, *e*, or *f* specific antiserum but were reactive with the antiserum to TW871. Based on these findings, we propose that these *S. mutans* strains be designated as new serotype *k*.

[0021] **Serotype distribution of 2500 strains from 50 subjects.** Table 3 shows the serotype distribution of 2500 recent isolates of *S. mutans*, of which 2450 were classified as *c*, *e*, or *f* type.

[0022] TABLE 3. Serotype distribution of 2500 *S. mutans* isolates obtained from 50 subjects

Serotype	Number of isolates	Number of subjects
c	1769	38
e	551	13
f	130	4
k	50	1

[0023] On the other hand, the RR extracts of the other 50 strains (YK1 through YK50) from a single subject (a girl with Down's syndrome) produced a precipitation band with antiserum to TW871, which also fused to the precipitation band of RR extracts of TW295, TW871, FT1, and SU1. All of these strains were considered to be serotype *k*. Table 4 shows the serotype distribution patterns in individual subjects, most of who were found to possess serotype *c* of *S. mutans* alone, followed by serotype *e* alone, while 5 of the 50 subjects possessed multiple serotypes.

[0024] TABLE 4. Individual serotype distribution among 50 subjects

Serotype	Number of subjects
single serotype	
c	34
e	8
f	2
k	1
multiple serotype	
c and *e*	3
e and *f*	1
c, *e*, and *f*	1

[0025] **Biological and serological characterization of a *gluA*-inactivated mutant.** A *gluA*-inactivated mutant strain, MT8148GD, showed typical biological features of *S. mutans* including rough colonies on MS agar, positive profiles of sugar fermentation, expressions of GTFs and PA, and high levels of sucrose-dependent adhesion and

cellular hydrophobicity. The RR extract of MT8148GD produced a precipitation band with the antiserum to TW871, however, not with serotype *c* specific antiserum, and fused to precipitation bands of TW295, TW871, FT1, SU1, and YK1.

[0026] **Phagocytosis assay.** The phagocytosis rate of strain MT8148GD was 22.0 ±2.4%, which was significantly lower than the parent strain MT8148 (68.4±4.1%) (P<0.001). Further, oral isolates of NN2001 (*c*), NN2002 (*e*), and NN2003 (*f*) showed a similar phagocytosis rate to MT8148, while the serotype *k* oral isolate strains FT1, SU1, and YK1 also showed a lower phagocytosis rate than MT8148 (P<0.001). In addition, the phagocytosis rates of all 4 blood isolate strains were significantly lower than those of the oral isolates (P<0.001), while that of MT8148GD, FT1, and TW295 was significantly lower than MT8148 until 60 min of incubation (P<0.001), and that of TW295 was also significantly lower than MT8148 after 90 min of incubation (P<0.001).

[0027] **DISCUSSION**

[0028] The RR extracts of 2 blood isolates (TW295 and TW871), 152 oral isolates (FT1 through FT51, SU1 through SU51, and YK1 through YK50), and a *gluA*-inactivated strain (MT8148GD) produced precipitation bands with antisera to TW295 or TW871. These isolates were shown to possess biological properties typical of *S. mutans*, including high levels of sucrose-dependent adhesion and hydrophobicity, and glucosyltransferases, while culture supernatants contained a 190 kDa protein antigen (PA) that produced precipitation bands with antiserum to PA of MT8148 (data not shown). On the other hand, the serological properties of these strains were different from those of the known *S. mutans* serotypes (*c*, *e*, and *f*) and, based on our findings, we propose a new *S. mutans* serotype *k*.

[0029] The isolation frequencies of *S. mutans* serotypes *c*, *e*, and *f* in the present study were closely similar to those reported in a Denmark study (16). In that report, 1 of 76 mutans streptococci from human dental plaque samples and 7 of 70 from human carious lesions, could not be serologically classified as serotype *a* through *g*. A biochemical analysis revealed that these untypable strains belonged to *S. mutans*, while

there was no description of the structures of the serotype specific polysaccharide. In another study, all 1047 *S. mutans* or *S. sobrinus* isolates taken from human dental plaque, carious dentin, or feces samples were able to be classified as serotype *c*, *e*, *f*, *d*, or *g* (5). Further, in a recent study conducted in another area of Japan, 144 strains of *S. mutans* or *S. sobrinus* from dental plaque were classified as serotype *c*, *e*, *d*, or *g*, and there were no serotype *f* strains or untypable strains detected (8). On the other hand, 9 out of 103 subjects were reported to harbor serologically untypable *S. mutans* in Japan recently, although precise description of the properties of the serotype specific polysaccharide was not described (7). Taken together, we speculated that serotype *k* organisms have appeared in Japan only recently.

[0030] In the present study, *S. mutans* strains with this new serotype were found in plaque samples from 3 children, with a total of 152 classified as serotype *k*. The origin of these clinical isolates has not yet been identified, however, serotype *k* strains occupied the majority of the *S. mutans* strains found in dental plaque samples from those 3 subjects, and all showed high levels of cellular hydrophobicity and sucrose-dependent adherence, as well as lower rates of phagocytosis. These findings suggest that serotype *k* strains of *S. mutans* are present in the oral cavity of humans, because of their high hydrophobicity and sucrose-dependent adhesion, along with the expressions of GTFs and PA, and may be able to survive in blood due to their lower phagocytotic capability. Therefore, special care must be taken for children with heart disorders. In particular, it may be more risky for Down's syndrome patients with *S. mutans* serotype *k* strain in the oral cavity, as they have been reported to have a possible congenital basis for disorders of PMN functions (13), and are also known to be susceptible to ventricular septal defect (11). As detection of new serotype *k* in these patients may indicate an increased risk, a clinical approach such as antibiotics prescription prior to dental treatment may be required and should be kept in mind.

[0031] Infective endocarditis is known to be initiated by an invasion of pathogenic bacteria into the bloodstream, however, the mechanisms of invasion and survival of *S. mutans* in blood have not yet been clarified. In the present study, we found that the

phagocytosis rate of the glucose side chain defective isogenic mutant strain was lower than that of the parent strain. In addition, the serotype *k* oral and blood isolates presented similar capabilities. Together, these findings suggest that serotype *k* strains, which are considered to lack a glucose side chain in serotype specific polysaccharide, may be possible bacteremia pathogenic strains. On the other hand, the blood isolate strains TW964 (*e*) and TW1378 (*f*) were less susceptible to phagocytosis than the oral isolates NN2002 (*e*) and NN2003 (*f*), though we have shown that TW964 lacks a glucan-binding protein A (15), whereas any such alteration of TW1378 has not been found. Thus, there may be an alteration of cell surface structures other than the serotype specific polysaccharide that has an effect on phagocytic ability.

[0032] REFERENCES

1. Ajdic , D., *et al.* 2002. *Proc. Natl. Acad. Sci. USA* 99:14434-14439.
2. Bentley, R. W., et al. 1991. *Int. J. Syst. Bacteriol.* 41:487-494.
3. Coykendall, A. L. 1989. *Clin. Microbiol. Rev.* 2:315-328.
4. Fujiwara, T., et al. 2001. *Eur. J. Oral Sci.* 109:330-334.
5. Hamada, S., et al. 1980. *J. Clin. Microbiol.* 11:314-318.
6. Hamada, S., et al. 1981. *Infect. Immun.* 32:364-372.
7. Hirakawa, M., and K. Takada. 2003. *Caries Res.* 37:212-217.
8. Kozai, K., et al. 1999. *Microbiol. Immunol.* 43:99-106.
9. Linzer, R., et al. 1986. *In* S. Hamada, et al., (ed.), Molecular microbiology and immunology of *Streptococcus mutans*. Elsevier Science Publishers, Amsterdam, pp. 29-38.
10. Macrina, F. L., *et al.* 1983 *Gene* 25:145-150.
11. Marino, B. 1996. *In* B. Marino and S. M. Pueschel (ed.), Heart disease in persons with Down syndrome. Paul Brookes, Baltimore, pp. 133-140..
12. Masuda, N., *et al.* 1985. *Microbios.* 44: 223-232.
13. Mills, E. L., and P. G. Quie. 1980. *Rev. Infect. Dis.* 2:505-517.
14. Minami, T., et al. 1990. *Oral Microbiol. Immunol.* 5:189-194.
15. Nakano, K., et al. 2002. *J. Dent. Res.* 81:376-379.

16. Perch, B., et al. 1974. *Acta. Pathol. Microbiol. Scand. [B] Microbiol. Immunol.* 82:357-370.

17. Rosenberg, M., et al. 1980. *FEMS Microbiol. Lett.* 9:29-33.

18. Tobian, J. A., and F. L. Macrina. 1982. *J. Bacteriol.* 152:215-222.

19. Ullman R. F., et al. 1988. *Heart Lung* 17:209-212.

20. Vose J. M., et al. 1987. *Am. J. Med.* 82:630-632.

21. Yamashita, Y., et al. 1998. *Microbiology* 144:1235-1245.

[0033] EXAMPLE 2

[0034] Recently, a serotype specific polysaccharide was demonstrated to play important roles in streptococcal adherence to human monocytic and fibroblastic cells, and was speculated to be the most efficient cytokine-stimulating component (Engels-Deutsch *et al.*, 2003). However, the relationship between serotype specific polysaccharide and dental caries remains to be elucidated. The purpose of the present example was to analyze the role of the glucose-side chain of serotype specific polysaccharide in the cariogenicity of the newly elucidated serotype *k* strains of *S. mutans*.

[0035] MATERIALS & METHODS

[0036] *S. mutans* Strains

S. mutans strains used in the present example are listed in Table 4. An oral isolate strain of MT8148 (*c*) was selected from the stock culture collection in our laboratory (Minami *et al.*, 1990). Strains FT1 (*k*), SU1 (*k*), and YK1 (*k*) were also isolated from the oral cavity of Japanese children (Nakano *et al.*, 2004). In addition, strain YT1 was isolated from the oral cavities of a 6-year-old healthy boy, and confirmed to be *S. mutans* serotype *k* according to methods described previously (Nakano *et al.*, 2004). Strains MT8148R and YK1R were made resistant to streptomycin by repeated passages in increasing concentrations of the antibiotic, up to a final concentration of 1500 μg of streptomycin per mL of agar medium (Ooshima *et al.*, 2000). Strain MT8148GD, a *gluA*-inactivated isogenic mutant strain of MT8148, was constructed in our previous

study (Nakano *et al.*, 2004). Strains MT8148RGD and FT1GD, which had the *gluA* gene insertionally inactivated, were constructed from MT8148R and FT1 (serotype *k*), respectively, as described previously (Nakano *et al.*, 2004).

[0037] Table 4. Bacterial strains used in the present study

Strain	Serotype	Features	Reference
MT8148	*c*	Oral isolate from healthy child	Minami *et al.* (1990)
MT8148GD	*k*	Em^r; strain MT8148 carrying *emr* inserted into *gluA*	Nakano *et al.* (2004)
MT8148R	*c*	Sm^r; repeated passages of MT8148 in increasing concentrations of streptomycin	Ooshima *et al.* (2000)
MT8148RGD	*k*	Sm^r, Em^r; strain MT8148 carrying *emr* inserted into *gluA*	This study
TW295	*k*	Blood isolate from 59-year-old male with bacteremia following a tooth extraction procedure	Fujiwara *et al.* (2001)
TW871	*k*	Blood isolate from 45-year-old female with infective endocarditis complicated with subarachnoid hemorrhage	Fujiwara *et al.* (2001)
FT1 (NN2011)	*k*	Oral isolate from 3-year-old healthy girl	Nakano *et al.* (2004)
FT1GD	*k*	Em^r; strain FT1 carrying *emr* inserted into *gluA*	This study
SU1 (NN2029)	*k*	Oral isolate from 10-year-old healthy girl	Nakano *et al.* (2004)
YK1	*k*	Oral isolate from 6-year-old girl with Down's syndrome	Nakano *et al.* (2004)
YK1R	*k*	Sm^r; repeated passages of YK1 in increasing concentrations of streptomycin	This study
YT1	*k*	Oral isolate from 6-year-old healthy boy	This study

Em^r: erythromycin resistant, Sm^r: streptomycin resistant

[0038] *In Vitro* Analysis of Cariogenic Properties

S. mutans sucrose-dependent adhesion to glass tubes and sucrose-independent adhesion to saliva-coated hydroxyapatite (SHA) were analyzed as described previously (Ooshima *et al.* 2001; Nakano *et al.* 2002). Cellular hydrophobicity and dextran-binding activity were also measured using a method described previously (Fujiwara *et al.*, 2001; Nakano *et al.*, 2002).

[0039] Expression of GTFs

Immunoblotting of GTFs was performed to assess the amount of expressed protein using cell-associated (CA)- and cell-free (CF)-GTF antisera, which were generated in our previous study (Minami *et al.*, 1990). The test organisms were grown in Brain Heart Infusion (BHI) broth (Difco Laboratories, Detroit, MI, USA) at 37℃ to an optimal density of 1.0 at 550 nm. The bacterial cells and supernatant, concentrated by ammonium sulfate precipitation, were dissolved in SDS gel loading buffer. An equal amount of each protein was separated by 7% SDS polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA, USA). The transferred protein bands were reacted with rabbit antibodies against CA- or CF-GTF, and then visualized using an alkaline phosphatase-conjugated anti-rabbit immunoglobulin G antibody (New England Biolabs, Beverly, MA, USA) and 5-bromo-4-chrolo-3-indolylphosphate-nitroblue tetrazolium substrate (Moss Inc., Pasadena, MD, USA). In addition, the ratio of the intensity of the expression of GTFB to that of GTFC in each strain was estimated by relative densitometric analysis of the reaction bands using NIH image software (National Technical Information Service, Springfield, VA, USA).

[0040] Measurement of CA- and CF-GTF Activities

CA- and CF-GTF activities were estimated by [^{14}C] glucan synthesis from [^{14}C] sucrose, using a method described previously (Matsumoto *et al.*, 2003). One unit of GTF activity was defined as the amount of enzyme needed to incorporate 1μ mol of glucose residue from sucrose into glucan in 1 minute.

[0041] Caries Inducing Experiments in Rats

All animal procedures and protocols were approved by the Animal Committee of Osaka University Graduate School of Dentistry. Caries-inducing activities were examined in 36 specific pathogen-free (SPF) Sprague-Dawley rats (12 rats per group) (Charles River Inc., Osaka, Japan) according to a method described previously (Nakano *et al.*, 2002). The 3 tested strains (MT8148R, MT8148RGD, and YK1R) at 5x10^9 CFU were

individually orally infected into rats aged 18 to 22 days, after which plaque and caries scores for each rat were evaluated at the age of 72 days.

[0042] Statistical Analysis

Intergroup differences of various factors were estimated by a statistical analysis of variance (ANOVA) for factorial models. Fisher's protected least-significant difference test was used to compare individual groups.

[0043] RESULTS

[0044] Table 5 shows the biological properties from the *in vitro* analysis of cariogenicity. MT8148GD showed a significantly lower rate of sucrose-dependent adhesion to a glass surface, sucrose-independent adhesion to SHA, dextran-binding activity, CA-GTF activity, and CF-GTF activity ($P<0.001$), whereas there were no significant differences in these properties between FT1 and FT1GD. There were significant differences between MT8148 and the other strains by Fisher's PLSD analysis

[0045] Table 5. Biological properties of serotype *k* strains

Strains	Sucrose-dependent adhesion [Mean±SD (%)]	Sucrose-independent adhesion [Mean±SD (%)]	Dextran-binding activity(A405) [Mean±SD]	CA-GTF activity [Mean±SD mU/mL)]	CF-GTF activity [Mean±SD (mU/mL)]
MT8148 (*c*)	91.5±0.5	100.0±2.1	0.15±0.03	73.0±3.9	95.0±2.8
MT8148GD (*k*)	70.5±3.8[a]	88.6±1.7[a]	0.02±0.01[a]	29.4±0.8[a]	59.8±0.9[a]
TW295 (*k*)	70.4±3.1[a]	77.9±2.0[a]	0.03±0.01[a]	34.9±1.6[a]	25.1±0.3[a]
TW871 (*k*)	75.1±0.9[a]	56.0±3.8[a]	0.09±0.04[a]	61.2±1.5[a]	56.0±2.3[a]
FT1 (*k*)	83.1±2.1[a]	89.6±0.3[a]	0.05±0.00[a]	38.6±0.3[a]	35.7±0.7[a]
FT1GD (*k*)	81.8±0.4[a]	89.8±0.2[a]	0.05±0.01[a]	41.2±1.2[a]	38.0±1.1[a]
SU1 (*k*)	85.4±0.9[a]	105.9±4.1	0.07±0.02[a]	48.3±3.7[a]	33.0±0.2[a]
YK1 (*k*)	82.7±1.2[a]	95.0±3.4	0.03±0.00[a]	47.4±2.5[a]	38.4±0.6[a]
YT1 (*k*)	80.8±1.4[a]	87.7±9.7[a]	0.02±0.01[a]	28.9±1.7[a]	30.4±0.4[a]

([a] $P<0.001$).

[0046] The serotype *k* blood isolates TW295 and TW871, and the 3 oral isolates SU1, YK1, and YT1, each showed a significantly lower sucrose-dependent adhesion rate, dextran binding activity, CA-GTF activity, and CF-GTF activity than MT8148

(P<0.001). As for sucrose-independent adhesion to SHA, the rates of the tested serotype *k* strains except for strains SU1 and YK1 were significantly lower than that of MT8148 (P<0.001). In addition, there were no significant differences in cellular hydrophobicity between MT8148 and the other tested strains (data not shown).

[0047] GTFB, GTFC, and GTFD were found expressed in all of the tested strains, however, the intensity of GTFB was significantly stronger in MT8148 than in any of the other strains. The ratio of GTFB expression intensity to that of GTFC of MT8148 was 1.80±0.15, which was significantly higher than that of MT8148GD (0.80±0.11) and those of the other serotype *k* blood or oral isolates (ratios ranging from 0.17-0.81) (P<0.001). In contrast, there were no significant differences in GTFD expression intensity among all the tested strains.

[0048] In the rats, serotype *k* induced evident dental caries. However, there were no significant differences in caries scores or plaque index between strain MT8148R, its mutant MT8148RGD, and oral isolate YK1R (Table 3).

[0049] **DISCUSSION**

The serotype *k* blood isolate TW871 has been shown to have extremely low levels of cariogenicity both *in vitro* and *in vivo* as compared to reference strain MT8148 (Nakano *et al.*, 2002). In our following study, another serotype *k* blood isolate, TW295, induced evident dental caries in rats, however, the caries scores in infected animals were significantly lower than those in MT8148R infected rats (unpublished data). These results led us to consider that serotype *k* strains might lower levels of cariogenicity than MT8148. In the present results, significant differences in several *in vitro* properties regarding cariogenicity between MT8148 and MT8148GD as well as other serotype *k* clinical isolates (Table 2) were seen, however, the serotype *k* oral isolate YK1R showed no significant caries-inducing difference in rats as compared to MT8148R (Table 6).

[0050] Table 6. Caries inducing activity of serotype *k* strains in rats

Strains	Number of rats	Plaque Index [Mean±SE]	Caries Scores [Mean±SE] Smooth	Total
MT8148R (*c*)	12	0.9±0.1	13.0±0.8	55.0±2.8
MT8148RGD (*k*)	12	0.9±0.1	13.4±1.6	53.6±5.3

Strains	Number of rats	Plaque Index [Mean±SE]	Caries Scores [Mean±SE]	
			Smooth	Total
YK1R (*k*)	12	0.8±0.1	14.5±1.5	54.1±4.6

[0051] The *in vivo* experiment using SPF rats for analysis of the cariogenicity of GTF-defective mutant strains revealed significant lower smooth surface caries scores for rats infected with the GTFB- or GTFC-defective mutant strain than for those infected with the parent strain, however, the smooth surface caries scores of rats infected with a GTFD-defective mutant were as high as those infected with the parent strain in a previous report (Yamashita *et al.*, 1993). In addition, our previous study results indicated that the smooth surface caries scores of rats infected with a GbpA- or GbpC-defective mutant strain were not significantly different from those infected with the parent strain (Matsumura *et al.*, 2003). Sucrose-dependent adhesion to a glass surface by GTF- or Gbp-defective mutant strains has also been analyzed, and the rates of GTFB-, GTFC-, GTFD-, GbpA-, and GbpC-defective mutant strains were sown to be 26%, 12%, 59%, 71%, and 65%, respectively (Ooshima *et al.*, 2001; Matsumura *et al.*, 2003). Those findings indicate that mutant strains with a drastic reduction of sucrose-dependent adhesion, such as GTFB- or GTFC-defective mutant may have significantly lower caries-inducing activity on smooth tooth surfaces *in vivo*. On the other hand, the sucrose-dependent adhesion rate of MT8148GD in the present study was 70.5%, which was significantly lower than the parent strain (Table 2), while the adhesion rates of the present serotype *k* oral and blood isolates ranged from 70.4% to 85.5%, which were significantly lower than the 50 serotype *c* oral isolates of *S. mutans* tested (data not shown). Therefore, a defect of the glucose side chain in serotype specific polysaccharide may be associated with the lower rate of sucrose-dependent adhesion seen *in vitro*, whereas the defect itself may not cause a significant reduction of caries *in vivo*.

[0052] In the present study, the biological properties of MT8148GD, constructed by insertional inactivation of the *gluA* gene, which encodes the enzyme that catalyzes the production of UDP-D-glucose, the immediate precursor of the glucose side chain donor,

were compared with those of the parent strain MT8148. Since UDP-D-glucose is reported to be important for the viability of *S. mutans* in a low pH environmental condition (Yamashita *et al.*, 1998), we speculated that inactivation of the *gluA* gene itself may cause an alteration of the properties relating to cariogenicity. Therefore, we constructed an additional mutant strain of serotype *k* strain FT1 (FT1GD), in which the *gluA* gene was insertionally inactivated. Our results showed that there were no significant differences in the biological properties between FT1 and FT1GD (Table 2), indicating that inactivation of the *gluA* gene itself is not associated with the properties measured in the *in vitro* assay. Therefore, we concluded that the presence of the glucose side chain of the serotype specific polysaccharide would be important for higher sucrose-dependent adhesion, sucrose-independent adhesion, dextran-binding activity, and CA- and CF-GTF activities.

[0053] A reduction of CA- and CF-GTF activity was prominent in the serotype *k* clinical isolates, as well as in MT8148GD (Table 5). Further, the CA- and CF-GTF activities in the serotype *k* clinical isolates were significantly lower than those in 30 clinical isolates of *S. mutans* (16 serotype *c* strains, 8 serotype *e* strains, and 6 serotype *f* strains) (data not shown). The optimal GTFB/GTFC/GTFD ratio necessary for appropriate colonization in vitro was determined in our previous study (Ooshima *et al.*, 2001), and deviation from this ratio could compromise the adherence properties and structure of plaque biofilm (Idone *et al.*, 2003). Western blot analysis in the present study indicated that the expression of GTFB tended to be lower in the serotype *k* strains than in MT8148. GTFB is thought to be responsible for the majority of water-insoluble glucan synthesis, which is an important virulence factor in initial caries development, as it caused an increased level of adherence and accumulation of mutans streptococci in the plaque of young children (Matto-Graner *et al.*, 2000). Thus, the lower CA-GTF activity of the serotype *k* strains may be attributable to the lower expression of GTFB, while its localization on the cell surface might be correlated with the presence of a glucose side chain of serotype specific polysaccharide. Taken together, these results suggest that a defect of glucose side chain in the serotype specific

polysaccharide of *S. mutans* may be associated with cariogenicity of the organism, though to a lesser extent than the other major surface structures of *S. mutans*, such as GTFs, PA and Gbps.

[0054] REFERENCES

Crowley PJ, et al. (1999). *Infect Immun* 67:1201-1206.

Engels-Deutsch M, et al. (2003). *Infect Immun* 71:5169-5177.

Fujiwara T, et al. (2001). *Eur J Oral Sci* 109:330-334.

Hamada S, and Slade HD (1980). *Microbiol Rev* 44:331-384.

Idone V, et al. (2003). *Infect Immun* 71:4351-4360.

Koga T, et al. (1990). *Infect Immun* 58:289-296.

Linzer R, et al. (1986). In: Molecular microbiology and immunology of *Streptococcus mutans*. Hamada S, et al. ed. Amsterdam: Elsevier Science Publishers, pp. 29-38.

Mattos-Graner RO, et al. (2000). *J Dent Res* 79:1371-1377.

Matsumoto M, et al. (2003). *FEMS Microbiol Lett* 228:73-80.

Matsumura M, et al. (2003). *Microbiol Immunol* 47:213-215.

Minami T, et al. (1990). *Oral Microbiol Immunol* 5:189-194.

Nakano K, et al. (2002). *J Dent Res* 81:376-379.

Nakano K, et al. (2004). *J Clin Microbiol* (in press)

Ooshima T, et al. (2000). *Arch Oral Biol* 45:639-645.

Ooshima T, et al. (2001). *J Dent Res* 80:1672-1677.

Yamashita Y, et al. (1993). *Infect Immun* 61:3811-3817.

Yamashita Y, et al. (1998). *Microbiology* 144:1235-1245.

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TW295 (k)       ATGAAAAGACTACTTTTGTATGTGCATTTTAATAAATATAATCGTGTGAGTTCTCATGTT
TW871 (k)        ATGAAAAGACTACTTTTGTATGTGCATTTTAATAAATATAATCGTGTGAGTTCTCATGTT
FT1 (k)         ATGAAAAGACTACTTTTGTATGTGCATTTTAATAAATATAATCGTGTGAGTTCTCATGTT
YT1 (k)         ATGAAAAGACTGCTTTTGTATGTGCATTTTAATAAATATAATCGTGTGAGTTCCCATGTT
UA159 (c)       ATGAAGCGCCTGCTTTTATATGTTCATTTTAATAAATACAATCGGGTAAGTTCCCATGTC
Xc (c)          ATGAAGCGCCTGCTTTTATATGTTCATTTTAATAAATACAATCGGGTAAGTTCCCATGTC
MT8148 (c)      ATGAAGCGCCTGCTTTTATATGTTCATTTTAATAAATACAATCGGGTAAGTTCCCATGTC
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TW295           TACTACCAACTAACACAAATGCGCCCCTTATTTTCAAGAGTAGTTTTCATCACAAATAGT
TW871           TACTACCAACTAACACAAATGCGCCCCTTATTTTCAAGAGTAGTTTTCATCACAAATAGT
FT1             TACTACCAACTAACACAAATGCGCCCCTTATTTTCAAGAGTAGTTTTCATCACAAATAGT
YT1             TACTACCAACTGACACAAATGCGCCCCTTATTTTCAAGAGTAGTTTTCATCACAAATAGC
UA159           GTTTATCAGTTGACTCAAATGAGATCCTTGTTTTCAAAAGTTATCTTTATTTCAAATAGC
Xc              GTTTATCAGTTGACTCAAATGAGATCCTTGTTTTCAAAAGTTATCTTTATTTCAAATAGC
MT8148          GTTTATCAGTTGACTCAAATGAGATCCTTGTTTTCAAAAGTTATCTTTATTTCAAATAGC
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TW295           CACCTATCTCAGGAGAACCAAGATAAGCTGCGCAGTCAAAAGTTGATGGATGATTTTCTA
TW871           CACCTATCTCAGGAGAACCAAGATAAGCTGCGCAGTCAAAAGTTGATGGATGATTTTCTA
FT1             CACCTATCTCAGGAGAACCAAGATAAGCTGCGCAGTCAAAAGTTGATGGATGATTTTCTA
YT1             CATCTAGCTCAGGAGGACCAAGACAAGCTGCGCAATCAAAATTTGATGGATGATTTTCTA
UA159           CAAGTGGCAGATGCGGATGTCAAAATGCTAAGAGAAAAGCATCTCATTGATGACTTCATT
Xc              CAAGTGGCAGATGCGGATGTCAAAATGCTAAGAGAAAAGCATCTCATTGATGACTTCATT
MT8148          CAAGTGGCAGATGCGGATGTCAAAATGCTAAGAGAAAAGCATCTCATTGATGACTTCATT
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TW295           CAGAGGGAAAATATCGGTTTTGATTTTGCAGCTTGGCGTGATGGGATGAATCACATTGGT
TW871           CAGAGGGAAAATATCGGTTTTGATTTTGCAGCTTGGCGTGATGGGATGAATCACATTGGT
FT1             CAGAGGGAAAATATCGGTTTTGATTTTGCAGCTTGGCGTGATGGGATGAATCACATTGGT
YT1             CAGAGAGAAAATATCGGTTTTGATTTTGCGGCTTGGCGTGATGGGATGAATCACATTGGC
UA159           CAACGGCAGAATTCTGGATTTGACTTTGCAGCTTGGCGAGATGGAATGGTCTTTGTCGGT
Xc              CAACGGCAGAATTCTGGATTTGACTTTGCAGCTTGGCGAGATGGAATGGTCTTTGTCGGT
MT8148          CAACGGCAGAATTCTGGATTTGACTTTGCAGCTTGGCGAGATGGAATGGTCTTTGTCGGT
                **  *  * ***   ** ***** ***** ******** ***** ***       * **

TW295           TTTGACAATCTTGATTCTTATGATATTGTCACTGTTATGAACGATACTTGCTTTGGACCT
TW871           TTTGACAATCTTGATTCTTATGATATTGTCACTGTTATGAACGATACTTGTTTTGGACCT
FT1             TTTGACAATCTTGATTCTTATGATATTGTCACTGTTATGAACGATACTTGCTTTGGACCT
YT1             TTTGACAATCTTGATTCTTATGATATTGTCACTGTTATGAACGATACTTGCTTTGGACCT
UA159           TTTGATGAACTTGTGACATATGACTCGGTAACAACCATGAATGACACTTGTTTTGGACCT
Xc              TTTGATGAACTTGTGACATATGACTCGGTAACAACCATGAATGACACTTGTTTTGGACCT
MT8148          TTTGATGAACTTGTGACATATGACTCGGTAACAACCATGAATGACACTTGTTTTGGACCT
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TW295           TTGTGGGATGTTAAGGAGCATTACCTGTCCTATGAAAAGCAGGACGA-GATTGATTTTTG
TW871           TTGTGGGATGTTAAGGAGCATTACCTGTCCTATGAAAAGCAGGACGA-GATTGATTTTTG
FT1             TTGTGGGATGTTAAGGAGCATTACCTGTCCTATGAAAAGCAGGACGA-GATTGATTTTTG
YT1             TTGTGGGATGTTAAGGATTATTACCTGTCCTATGAAAAGCAAGATGA-AGTTGATTTTTG
UA159           CTTTGGGAAATGTATTCAATTTATCAAGAATTTGAAAC-CAAGACGACAGTTGATTTTTG
Xc              CTTTGGGAAATGTATTCAATTTATCAAGAATTTGAAAC-CAAGACGACAGTTGATTTTTG
MT8148          CTTTGGGAAATGTATTCAATTTATCAAGAATTTGAAAC-CAAGACGACAGTTGATTTTTG
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TW295       GGGATTGACTAATAATCGTGCGACTAAGCAGTTTAAGGAACATATTCAAAGCTACTTTAT
TW871       GGGATTGACTAATAATCGTGCGACTAAGCAGTTTAAGGAACATATTCAAAGCTACTTTAT
FT1         GGGATTGACTAATAATCGTGCGACTAAGCAGTTTAAGGAACATATTCAAAGCTACTTTAT
YT1         GGGATTGACCAATAATCGTGCGACTAAGCAGTTTAAGGAGCATATTCAAAGCTACTTTAT
UA159       GGGATTGACCAACAACCGTGCAACCAAGTCATTTCGTGAGCATATTCAAAGTTACTTTAT
Xc          GGGATTGACCAACAACCGTGCGACCAAGTCATTTCGTGAGCATATTCAAAGTTACTTTAT
MT8148      GGGATTGACTAATAATCGTGCGACTAAGCAGTTTAAGGAACATATTCAAAGCTACTTTAT
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TW295       TACCTTTAAAAAAGCTGTTATTCAATCGGAGGCCTTTCATAATTTTTGGGAGAACATCCA
TW871       TACCTTTAAAAAAGCTGTTATTCAATCGGAGGCCTTTCATAATTTTTGGGAGAACATCCA
FT1         TACCTTTAAAAAAGCTGTTATTCAATCGGAGGCCTTTCATAATTTTTGGGAGAACATCCA
YT1         TACTTTTAAAAAGGCTGTTATTCAATCAAATGCCTTTCATGATTTTTGGGAGAATATCCA
UA159       TTCATTTAAAGCATCTGTTTTAAGAAGCACCGCTTTCAGAGACTTTTGGGAAAATATAAA
Xc          TTCATTTAAAGCATCTGTTTTAAGAAGCACCGCTTTCAGAGACTTTTGGGAAAATATAAA
MT8148      TTCCTTTAAAAAAGCTGTTATTCAATCGGAGGCCTTTCATAATTTTTGGGAGAACATCCA
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TW295       AAATCATGCAGATATTCAACGTGTCATTGATGATTACGAAACTCAGGTGACGACAACGCT
TW871       AAATCATGCAGATATTCAACGTGTCATTGATGATTACGAAACTCAGGTGACGACAACGCT
FT1         AAATCATGCAGATATTCAACGTGTCATTGATGATTACGAAACTCAGGTGACGACAACGCT
YT1         AAATCATACAGATGTTCAGCGTGTCATTGATGATTATGAAACTCAGGTGACGACGACACT
UA159       AGAGTATCAGGATGTTCAAAAGGTGATTGATCAGTATGAAACAAAGGTCACGACAACTCT
Xc          AGAGTATCAGGATGTTCAAAAGGTGATTGACCAGTATGAAACAAAAGTCACGACAACTCT
MT8148      AAATCATGCAGATATTCAACGTGTCATTGATGATTACGAAACTCAGGTCACGACAACTCT
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TW295       TTTGGATGCTGGTTTTCAATATGATGTCGTTTTTGATACGACCAAAGAAGATGCTTCGCG
TW871       TTTGGATGCTGGTTTTCAATATGATGTCGTTTTTGATACGACCAAAGAAGATGCTTCGCA
FT1         TTTGGATGCTGGTTTTCAATATGATGTCGTTTTTGATACGACCAAGGAAGATGCTTCGCA
YT1         TCTGGATGCAGGTTTTAAGTATAGTGTCATATTTGACACAACCAAAGAAGATGCTTCACA
UA159       CTTAGATGCTGGTTTTCAATATGATGTCGTTTTTGATACGACCAAGGAAGATGCTTCGCA
Xc          CTTAGATGCTGGTTTTCAATATGATGTCGTTTTTGATACGACCAAGGAAGATGCTTCGCA
MT8148      CTTAGATGCTGGTTTTCAATATGATGTCGTTTTTGATACGACCAAGGAAGATGCTTCGCA
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TW295       TATGCTTCATGCAGACTTCTCTTACTATAATCCAACAGCTATTTTGAATCATAGGGTGCC
TW871       TATGCTTCATGCCGACTTCTCTTACTATAATCCAACAGCTATTTTGAATCATAGGGTGCC
FT1         TATGCTTCATGCAGACTTCTCTTACTATAATCCAACAGCTATTTTGAATCATAGGGTGCC
YT1         TATGCTGCATGCAGATTTTTCTTATTATAATCCAACAGCTATTTTGAACCATAGAGTGCC
UA159       TATGCTTCATGCAGACTTCTCTTACTATAATCCAACAGCTATTTTGAATCATAGGGTGCC
Xc          TATGCTTCATGCAGACTTCTCTTACTATAATCCAACAGCTATTTTGAATCATAGGGTGCC
MT8148      TATGCTTCATGCAGACTTCTCTTACTATAATCCAACAGCTATTTTGAATCATAGGGTGCC
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TW295       CTTTATCAAGGTTAAAGCGATTGACAATAATCAACATATTACGCCCTATCTTTTAAATGA
TW871       CTTTATCAAGGTTAAAGCGATTGACAATAATCAACATATTACGCCCTATCTTTTAAATGA
FT1         CTTTATCAAGGTTAAAGCGATTGACAATAATCAACATATTACGCCCTATCTTTCAAATGA
YT1         TTTTATCAAGGTTAAAGCTATTGATAATAATCAGCATATTACCCCCTACCTTTTAAATGA
UA159       CTTTATCAAGGTTAAAGCGATTGACAATAATCAACATATTACGCCCTATCTTTTAAATGA
Xc          CTTTATCAAGGTTAAAGCGATTGACAATAATCAACATATTACGCCCTATCTTTTAAATGA
MT8148      CTTTATCAAGGTTAAAGCGATTGACAATAATCAACATATTACGCCCTATCTTTTAAATGA
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TW871       TATTCAAAAGAATTCGACCTATCCTATTGATTTAATCGTTTCGCACATGTCAGAAATCAA
FT1         TATTCAAAAGAATTCGACCTATCCTATTGATTTAATCGTTTCGCACATGTCAGAAATCAA
YT1         TATTCAAAATCATTCGACCTATCCTATTGATTTAATCGTTTCTCACATGTCAGAAATCAA
UA159       TATTCAAAAGAATTCGACCTATCCTATTGATTTAATTGTTTCGCACATGTCAGAAATCAA
Xc          TATTCAAAAGAATTCGACCTATCCTATTGATTTAATTGTTTCGCATATGTCAGAAATCAA
MT8148      TATTCAAAAGAATTCGACCTATCCTATTGATTTAATTGTTTCGCACATGTCAGAAATCAA
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TW295       TTATCCTGATTTTAGTTATTTATTGGGTCACAAATATGTCAAGAAAAGGGAAAGAGTTGA
TW871       TTATCCTGATTTTAGTTATTTATTGGGTCACAAATATGTCAAGAAAAGGGAAAGAGTTGA
FT1         TTATCCTGATTTTAGTTATTTATTGGGTCACAAATATGTCAAGAAAAGGGAAAGAGTTGA
YT1         TTATCCTGATTTTAGTTACTTGTTGGGTCATAAATATGTCAAGAGAAAAGAAGCGGTTGA
UA159       TTATCCTGATTTTAGTTATTTATTGGGTCACAAATATGTCAAGAAAAGAGAAAGAGTTGA
Xc          TTATCCTGATTTTAGTTATTTATTGGGTCACAAATATGTCAAGAAAAGAGAAAGAGTTGA
MT8148      TTATCCTGATTTTAGTTATTTATTGGGTCACAAATATGTCAAGAAAAGAGAAAGAGTTGA
            ****************** ** ******** ************* **  ***   *****

TW295       TTTAAAGAATCAAAAAGCTGCGGTTCATCTCCATGTGTTTTATGTGGATTTGCTGGAAGA
TW871       TTTAAAGAATCAAAAAGCTGCGGTTCATCTCCATGTGTTTTATGTGGATTTGCTGGAAGA
FT1         TTTAAAGAATCAAAAAGCTGCGGTTCATCTCCATGTGTTTTATGTGGATTTGCTGGAAGA
YT1         TTTAACGGGTCAAAAAATTGCAGTTCATCTCCATGTTTTTTATGTGGATCTGCTAGAAGA
UA159       TTTAAAGAATCAAAAAGCTGCGGTTCATCTCCATGTGTTTTATGTGGATTTGCTGGAAGA
Xc          TTTAAAGAATCAAAAAGTTGCGGTTCATCTCCATGTGTTTTATGTGGATTTACTGGAAGA
MT8148      TTTAAAGAATCAAAAAGTTGCGGTTCATCTCCATGTGTTTTATGTGGATTTACTGGAAGA
            ***** *  *******  *** ************** ************ * ** *****

TW295       ATTTTTAACGGCATTTAAACAATTTCATTTTTCTTATGATTTATTTATAACGACAGATAG
TW871       ATTTTTAACGGCATTTAAACAATTTCATTTTTCTTATGATTTATTTATAACGACAGATAG
FT1         ATTTTTAACGGCATTTAAACAATTTCATTTTTCTTATGATTTATTTATAACGACAGATAG
YT1         ATTTTTGACAGCATTCAAGCAATTTCATTTTTCTTATGATTTATTTATGACAACAGATAG
UA159       ATTTTTAACGGCATTTAAGCAATTTCATTTTTCTTATGATTTATTTATAACGACAGATAG
Xc          ATTTTTAACGGCATTTAAGCAATTTCATTTTTCTTATGATTTATTTATAACGACAGATAG
MT8148      ATTTTTAACGGCATTTAAGCAATTTCATTTTTCTTATGATTTATTTATAACGACAGATAG
            ****** ** ***** ** ****************************** ** ********

TW295       TGATGATAAGAAAGCTGAAATTGAAGAGATTCTATCTGCAAACAGTCAAGAGGCTCAGGT
TW871       TGATGATAAGAAAGCTGAAATTGAAGAGATTCTATCTGCAAACAGTCAAGAGGCTCAGGT
FT1         TGATGATAAGAAAGCTGAAATTGAAGAGATTCTATCTGCAAACAGTCAAGAGGCTCAGGT
YT1         TGATGATAAGAAAGCTGAAATTGAAGAAATTCTAGCAGCAAATAATCAAGAAGTTCAGGT
UA159       TGATGATAAGAAAGCTGAAATTGAAGAGATTCTATCTGCAAACAGTCAAGAGGCTCAGGT
Xc          TGATGATAAGAAAGCTGAAATTGAAGAGATTCTATCCGCAAACGGTCAAGAAGCTCAGGT
MT8148      TGATGATAAGAAAGCTGAAATTGAAGAGATTCTATCTGCAAACAGTCAAGAAGCTCAGGT
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TW295       TTTTGTCACAGGCAATATTGGACGTGATGTTCTTCCTATGTTAAAATTAAAAAATTATTT
TW871       TTTTGTCACAGGCAATATTGGACGTGATGTTCTTCCTATGTTAAAATTAAAAAATTATTT
FT1         TTTTGTCACAGGCAATATTGGACGTGATGTTCTTCCTATGTTAAAATTAAAAAATTATTT
YT1         TTTTGTCACAGGGAATATTGGACGTGATGTTCTCCCTATGTTAAAATTAAAAAATTACTT
UA159       TTTTGTCACAGGCAATATTGGACGTGATGTTCTTCCTATGTTAAAATTAAAAAATTATTT
Xc          TTTTGTCACAGGCAATATTGGACGTGATGTTCTTCCTATGTTAAAATTAAAAAATTATTT
MT8148      TTTTGTCACAGGCAATATTGGACGTGATGTTCTTCCTATGTTAAAATTAAAAAATTATTT
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TW295           ATCTACCTATGATTTTGTTGGTCATTTTCATACCAAAAAGTCAAAGGAGGCTGATTTTTG
TW871           ATCTACCTATGATTTTGTTGGTCATTTTCATACCAAAAAGTCAAAGGAGGCTGATTTTTG
FT1             ATCTACCTATGATTTTGTTGGTCATTTTCATACCAAAAAGTCAAAGGAGGCTGATTTTTG
YT1             ATCTGCCTATGATTTTGTTGGCCATTTTCATACCAAAAAATCAAAAGAAGCTGATTTTTG
UA159           ATCTACCTATGATTTTGTTGGTCATTTTCATACCAAAAAGTCAAAGGAGGCTGATTTTTG
Xc              ATCTGCCTATGATTTTGTTGGTCATTTTCATACCAAAAAGTCAAAGGAGGCTGATTTTTG
MT8148          ATCTACCTATGATTTTGTTGGTCATTTTCATACCAAAAAGTCAAAGGAGGCTGATTTTTG
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TW295           GGCTGGCCAATCTTGGCGGGAAGAATTAATTGACATGTTGGTTAAACCAGCAGACAATAT
TW871           GGCTGGCCAATCTTGGCGGGAAGAATTAATTGACATGTTGGTTAAACCAGCAGACAATAT
FT1             GGCTGGCCAATCTTGGCGGGAAGAATTAATTGATATGTTGGTTAAACCAGCAGACAATAT
YT1             GGCTGGCCAATCTTGGCGGGAAGAATTAATTGACATGTTGGTTAAGCCAGCAGACAATAT
UA159           GGCTGGCCAATCTTGGCGGGAAGAATTAATTGACATGTTGGTTAAACCAGCAGACAATAT
Xc              GGCTGGCCAATCTTGGCGGGAAGAATTAATTGATATGTTGGTTAAACCAGCAGACAATAT
MT8148          GGCTGGCCAATCTTGGCGGGAAGAATTAATTGACATGTTGGTTAAACCAGCAGACAATAT
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TW295           TTTAGCGCAATTACAGCAAAACCCCAAAATTGGTTTGGTGATTGCTGATATGCCAACTTT
TW871           TTTAGCGCAATTACAGCAAAACCCAAAAATTGGTTTGGTGATTGCTGATATGCCAACTTT
FT1             TTTAGCGCAATTACAGCAAAACCCAAAAATTGGTTTGGTGATTGCTGATATGCCAACTTT
YT1             TTTAGCAGAATTACAGCAAAACCCGAAAATTGGTTTGGTTATTGCTGATATGCCAACTTT
UA159           TTTAGCGCAATTACAGCAAAACCCCAAAATTGGTTTGGTGATTGCTGATATGCCAACTTT
Xc              TTTAGCGCAATTACAGCAAAACCCAAAAATTGGTTTGGTGATTGCTGATATGCCAACTTT
MT8148          TTTAGCGCAATTACAGCAAAACCCAAAAATTGGTTTGGTGATTGCTGATATGCCAACTTT
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TW295           CTTTCGCTATAATAAAATTGTGGATGCTTGGAATGAACATTTGATTGCACCTGAGATGAA
TW871           CTTTCGCTATAATAAAATTGTGGATGCTTGGAATGAACATTTGATTGCACCTGAGATGAA
FT1             CTTTCGCTATAATAAAATTGTGGATGCTTGGAATGAACATTTGATTGCACCTGAGATGAA
YT1             CTTTCGCTATAATAAAATTGTTGATGCTTGGAATGAACATTTGATTGCACCTGAGATGAA
UA159           CTTTCGCTATAATAAAATTGTTGATGCTTGGAATGAACATTTGATTGCACCTGAGATGAA
Xc              CTTTCGCTATAATAAAATTGTTGATGCTTGGAATGAACATTTGATTGCACCTGAGATGAA
MT8148          CTTTCGCTATAATAAAATTGTTGATGCTTGGAATGAACATTTGATTGCACCTGAGATGAA
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TW295           TACATTATGGCAAAAGATGGGCATGACCAAAAAGATTGATTTCAATGCTTTTCACACTTT
TW871           TACATTATGGCAAAAGATGGGCATGACCAAAAAGATTGATTTCAATGCTTTTCACACTTT
FT1             TACATTATGGCAAAAGATGGGCATGACCAAAAAGATTGATTTCAATGCTTTTCACACTTT
YT1             TACACTATGGCAAGAGATGGGAATGACCAAAACGATTGATTTCAATGCTTTTCATACTTT
UA159           TACATTATGGCAAAAGATGGGCATGACCAAAAAGATTGATTTCAATGCTTTTCACACTTT
Xc              TACATTATGGCAAAAGATGGGCATGACCAAAAAGATTGATTTCAATGCTTTTCATACTTT
MT8148          TACATTATGGCAAAAGATGGGCATGACCAAAAAGATTGATTTCAATGCTTTTCATACTTT
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TW295           TGTCATGAGTTATGGCACTTTTGTTTGGTTTAAATATGATGCCTTAAAACCGCTCTTTGA
TW871           TGTCATGAGTTATGGCACTTTTGTTTGGTTTAAATATGATGCCTTAAAACCGCTCTTTGA
FT1             TGTCATGAGTTATGGCACTTTTGTTTGGTTTAAATATGATGCCTTAAAACCGCTCTTTGA
YT1             TGTCATGAGTTATGGCACTTTTGTTTGGTTTAAATATGATGCCTTAAAACCGCTCTTTGA
UA159           TGTCATGAGTTATGGCACTTTTGTTTGGTTTAAATATGATGCCTTAAAACCGCTCTTTGA
Xc              TGTCATGAGTTATGGTACTTTTGTTTGGTTTAAATATGATGCCTTAAAACCGCTCTTTGA
MT8148          TGTCATGAGTTATGGCACTTTTGTTTGGTTTAAATATGATGCCTTAAAACCGCTCTTTGA
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TW295           TTTAAATCTGACAGATGATGATGTGCCTGAGGAACCTTTACCGCAAAATTCTATTTTACA
TW871           TTTAAATCTGACAGATGATGATGTGCCTGAGGAACCTTTACCGCAAAATTCTATTTTACA
FT1             TTTAAATCTGACAGATGATGATGTGCCTGAGGAACCTTTACCGCAAAATTCTATTTTACA
YT1             TTTAAATCTGACAGATGATGATGTGCCTGAGGAACCTTTACCGCAAAATTCTATTTTACA
UA159           TTTAAATCTGACAGATGATGATGTGCCTGAGGAACCTTTGCCGCAAAATTCTATTTTACA
Xc              TTTAAATCTGACAGATGATGATGTGCCTGAGGAACCTTTACCGCAAAATTCTATTTTACA
MT8148          TTTAAATCTGACAGATGATGATGTGCCTGAGGAACCTTTACCGCAAAATTCTATTTTACA
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TW295           TGCTATTGAGCGTTTGCTGATCTACATTGCTTGGAATGAGCATTACGATTTTAGAATTTC
TW871           TGCTATTGAGCGTTTGCTGATCTACATTGCTTGGAATGAGCATTACGATTTTAGAATTTC
FT1             TGCTATTGAGCGTTTGCTGATCTACATTGCTTGGAATGAGCATTACGATTTTAGAATTTC
YT1             TGCTATTGAGCGTTTGCTGATCTACATTGCTTGGAATGAGCATTACGATTTTAGAATTTC
UA159           TGCTATTGAGCGTTTATTGATCTACATTGCTTGGAATGAGCATTACGATTTTAGAATTTC
Xc              TGCTATTGAGCGTTTGCTGATCTACATTGCTTGGAATGAGCATTACGATTTTAGAATTTC
MT8148          TGCTATTGAGCGTTTGCTGATCTACATTGCTTGGAATGAGCATTACGATTTTAGAATTTC
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TW295           TAAAAATCCAGTTGATCTGACGCCTTTCATAGATAATAAATTATTAAATAAACGTGGTAA
TW871           TAAAAATCCAGTTGATCTGACGCCTTTCATAGATAATAAATTATTAAATAAACGTGGTAA
FT1             TAAAAATCCAGTTGATCTGACGCCTTTCATAGATAATAAATTATTAAATAAACGTGGTAA
YT1             TAAAAATCCAGTTGATCTGACGCCTTTCATAGATAATAAATTATTAAATAAACGTGGTAA
UA159           TAAAAATCCAGTTGATCTGACGCCTTTCATAGATAATAAATTATTAAATGAACGTGGCAA
Xc              TAAAAATCCAGTTGATCTGACGCCTTTCATAGATAATAAATTATTAAATGAACGTGGTAA
MT8148          TAAAAATCCAGTTGATCTGACGCCTTTCATAGATAATAAATTATTAAATGAACGTGGCAA
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TW295           CTCAGCACCAAATACCTTTATTGATTTTAACCATATGGGAGGAATAAAAGGAGCTTTTAA
TW871           CTCAGCACCAAATACCTTTATTGATTTTAACCATATGGGAGGAATAAAAGGAGCTTTTAA
FT1             CTCAGCACCAAATACCTTTATTGATTTTAACCATATGGGAGGAATAAAAGGAGCTTTTAA
YT1             CTCAGCACCAAATACCTTTATTGATTTTAACCATATGGGAGGAATAAAAGGAGCTTTTAA
UA159           CTCAGCACCAAATACCTTTGTTGATTTTAACTATATGGGAGGAATAAAGGGAGCTTTTAA
Xc              CTCAGCACCAAATACCTTTGTTGATTTTAACTATATGGGAGGAATAAAAGGAGCTTTTAA
MT8148          CTCAGCACCAAATACCTTTGTTGATTTTAACTATATGGGAGGAATAAAGGGAGCTTTTAA
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TW295           GTATATCTTTATTGGTCCAGCTAGGGCTGTCAAATATATCCTTAAACGTTCTCTGCAAAA
TW871           GTATATCTTTATTGGTCCAGCTAGGGCTGTCAAATATATCCTTAAACGTTCTCTGCAAAA
FT1             GTATATCTTTATTGGTCCAGCTAGGGCTGTCAAATATATCCTTAAACGTTCTCTGCAAAA
YT1             GTATATCTTTATTGGTCCAGCTAGGGCTGTCAAATATATCCTTAAACGTTCTCTGCAAAA
UA159           ATATATTTTCATTGGTCCAGCTAGGGCTGTCAAATATATCCTGAAGCGTTCTCTGCAAAA
Xc              GTATATCTTTATTGGTCCAGCTAGGGCTGTCAAATATATCCTTAAACGTTCTCTGCAAAA
MT8148          ATATATTTTCATTGGTCCAGCTAGGGCTGTCAAATATATCCTTAAACGTTCTCTGCAAAA
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TW295           AATAAAGTCATGA
TW871           AATAAAGTCATGA
FT1             AATAAAGTCATGA
YT1             AATAAAGTCATGA
UA159           AATAAAGTCATGA
Xc              AATAAAGTCATGA
MT8148          AATAAAGTCATGA
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PCRのプロトコール

歯体より genomic DNA を抽出し,

genomic DNA 20ug/uL	5uL	
dNTP mixture	2uL	
10x PCR buffer	2uL	
Forward primer 20pmol/uL	1uL	
Reverse primer 20pmol/uL	1uL	
AmpliTaq Gold	0.1uL	
MilliQ water	8.9uL	Total 20uL

94C 5min
以下 30 cycles
94C 30sec
58C 30sec
72C 30sec
ここまで 30 cycles
72C 7min

2% Agarose S dissolved in TAE buffer で電気泳動

現在は，k 型の菌において特有の遺伝子配列の特定をすすめ，これを利用して患者プラークあるいは唾液から抽出した DNA から k 型のS. mutans を保有するヒトを簡便に特定する系の確立を進めています．以下がその経過です．

当初，ラムノース主骨格に結合するグルコース側鎖をコードする遺伝子である rgpE 遺伝子に変異が生じているのではないかという仮説のもと，実験を進めてきましたが，k type の各菌株においてこの遺伝子には変異がなりませんでした．一方で，ラムノース主骨格のラムノースの3つめから1つとばしの位置（5つめ，7つめ，．．．）のラムノースを結合することに関連するといわれる rgpF 遺伝子に関して k type の株で drastic な変異があり，かつそれが k type の株特有に共通していることがわかりました．そこで，ラムノースが2つめまで存在し（よってラムノースは検出される），グルコースが結合するラムノース は3つめ以降なのでグルコースは生成できるが結合されないから検出されない，との仮説のもと裏付けの実験を進めてもらっています（→このことについては貴社の前田先生に糖の分析を依頼させてもらっております）．

現在，TW295 (k), TW871 (k), FT1 (k), YT1 (k) および MT8148 (c) において rgp 付近の約25000の遺伝子配列を検討している途中ですが（約半分終わっております），これまでのところ k type において rgpF の配列以外に変異は認められておりません．また，並行して菌から抽出した染色体 DNA で c/e/f 特異的に増幅するプライマーおよび k 特異的に増幅するプライマーを作製し，それぞれで特異的に増幅する系が出来上がっております．近日中 に，患者プラークより分離したいろいろな菌の DNA の混ざったサンプルでも大丈夫か調べる予定です．